Immunology 2003 **109** 415–425

Differential regulation of interleukin-12 p40 and p35 induction via Erk mitogen-activated protein kinase-dependent and -independent mechanisms and the implications for bioactive IL-12 and IL-23 responses

HELEN S. GOODRIDGE*, WILLIAM HARNETT†, FOO Y. LIEW* & MARGARET M. HARNETT* **Division of Immunology, Infection and Inflammation, University of Glasgow, Glasgow, UK, and †Department of Immunology, University of Strathclyde, Glasgow, UK*

SUMMARY

Bioactive interleukin (IL)-12 is a 70 000-molecular weight (MW) heterodimeric cytokine comprising p40 and p35 chains. However, p40 can also form homodimers that antagonize bioactive IL-12 or heterodimerize with p19 to form IL-23, which exhibits overlapping yet distinct functions to that of IL-12. We now define distinct signalling mechanisms that regulate lipopolysaccharide (LPS)-mediated induction of IL-12 p40 and p35 in macrophages and which may therefore provide therapeutic targets for precise and specific fine-tuning of cytokine responses. Thus, whilst LPS-induced p38 mitogen-activated protein kinase (MAPkinase) activation is required for the induction of both p40 and p35 subunits, Erk MAPkinase signalling mediates negative feedback regulation of p40, but not p35, production. Such Erk MAPkinase activation is downstream of calcium influx and targets LPS-induced IL-12 p40 transcription by suppressing the synthesis of the transcription factor, interferon regulatory factor-1 (IRF-1). In contrast, negative regulation of the p35 subunit of IL-12 occurs via a calcium-dependent, but Erk-independent, mechanism, which is likely to involve nuclear factor (NF)-κB signalling. Finally, the importance of both Erk and p38 MAPkinases in differentially regulating IL-12 p40 and p35 production is underscored by each being targeted by ES-62, a product secreted by parasitic filarial nematodes to polarize the immune system towards an anti-inflammatory phenotype conducive to their survival.

INTRODUCTION

The production of interleukin (IL)-12 by phagocytes and antigen-presenting cells is critical for host defence against a variety of pathogens. IL-12 functions in both innate and adaptive immune mechanisms (reviewed in ref. 1), enhancing the phagocytic and bactericidal activities of phagocytes and the generation of other pro-inflammatory cytokines, as well as directing the development of type 1 T helper (Th1) responses. Bioactive IL-12 is a 70 000-molecular weight (MW) heterodimeric cytokine (p70) comprising disulphide-linked p40 and p35 subunits.

Received 3 April 2003; revised 13 May 2003; accepted 15 May 2003.

Correspondence: Dr M. M. Harnett, Division of Immunology, Infection and Inflammation, University of Glasgow, Western Infirmary, Dumbarton Road, Glasgow G11 6NT, UK. E-mail: M.Harnett@bio.gla.ac.uk

That p40 can also combine with p19, which is distantly related to p35, to form the recently identified IL-23[2] may explain, at least in part, why p40 protein is detected in vast excess over the p70 heterodimer. IL-12 and IL-23 have some overlapping and some distinct functions.[2,3] Both are capable of inducing proliferation and interferon-γ (IFN-γ) production by T cells, but while IL-12 preferentially acts on naïve T cells, IL-23 strongly activates memory T cells.[2] Further distinctions exist in their autocrine effects on dendritic cells (DCs). A recent report showed that immunoglobulin fusion proteins of both IL-12 and IL-23 induced IL-12 production from DCs, whereas IL-23–immunoglobulin, but not IL-12–immunoglobulin, acted directly on CD8α$^+$ DCs to promote immunogenic presentation of an otherwise tolerogenic tumour peptide.[3]

In addition to forming heterodimers with p19 and p35, p40 can also form homodimers with biological activity antagonistic to p70 heterodimers.[4,5] In contrast, neither p19 nor p35 homodimers have been reported. p35 has also been shown to dimerize with Epstein–Barr virus (EBV)-induced gene 3 (EBI-3),[6] but

the function of this heterodimer is as yet unknown. EBI-3 is also a component of the recently identified IL-27, forming heterodimers with a newly discovered p35-related protein, p28.[7] IL-27 drives rapid clonal expansion of naïve, but not memory, $CD4^+$ T cells and is strongly synergistic with IL-12 to induce IFN-γ production.

Macrophage (Mϕ) activation by pathogen products, such as lipopolysaccharide (LPS), results in the induction of pro-inflammatory cytokines, including IL-12. Induction of the p40 and p35 genes occurs following binding of LPS to its receptor complex, which includes lipopolysaccharide-binding protein (LBP), CD14 and Toll-like receptor-4 (TLR4) (reviewed in ref. 1). While the generation of p40 is predominantly regulated at the level of gene induction, regulation of p35 production occurs at both transcriptional and post-transcriptional levels, and includes the use of alternative transcription start sites and atypical post-translational processing mechanisms.[8–10] In contrast to p40, p35 gene expression is thought to require only presynthesized activators because its expression is not inhibited by treatment with cyclohexamide.[11] The promoter regions of both genes have been characterized and a number of transcription factor binding sites have been identified. Nuclear factor (NF)-κB and interferon regulatory factor (IRF) family members have been shown to contribute to IFN-γ priming and LPS induction of p40 and p35 gene expression (reviewed in ref. 1).

Although many of the molecular events relating to the induction of p35 and p40 mRNA transcription have been well characterized, little is known about the early, receptor proximal signalling mechanisms underlying LPS-mediated induction of IL-12 p40 and, in particular, IL-12 p35. We, and others, have previously shown that stimulation of Mϕs with LPS results in the activation of all three mitogen-activated protein kinase (MAPkinase) subfamilies.[12–15] We also demonstrated that LPS-induced IL-12 p40 production by Mϕs is dependent on p38 MAPkinase but negatively regulated by Erk MAPkinases at the level of transcription/message stability.[12] In this study, we have further dissected the signalling pathways that contribute to the regulation of IL-12 p40 and/or p70 generation. We show that calcium influx, which has been reported to be elicited following stimulation of Mϕs with LPS,[16,17] results in the enhanced activation of Erk MAPkinase and this targets IL-12 p40 transcription by suppressing the synthesis of the transcription factor, IRF-1. In contrast, negative regulation of the p35 subunit of IL-12 occurs via a calcium-dependent, but Erk-independent, mechanism. Moreover, we show that a pathogen product, ES-62, secreted by the filarial nematode *Acanthocheilonema viteae*, can effect modulation of the immune response to an anti-inflammatory phenotype by subverting such MAPkinase-dependent regulation of IL-12 p70 production.

Together our results show that distinct regulatory pathways control induction of the IL-12 p40 and p35 subunits required for generation of bioactive IL-12 p70 and that both of these can be modulated by a defined product of an infective pathogen. Powerful biological implications of these findings are that such differential regulatory circuits should allow therapeutic fine-tuning of the precise cytokine components required to elicit the particular phenotype – for example Th1 (bioactive IL-12), Th2/anti-inflammatory (antagonistic IL-12 p40 homodimers), $CD8\alpha^+$ DC or memory (IL-23) – of immune response necessary to combat infection by pathogens or pathology resulting from inflammatory disorders.

MATERIALS AND METHODS

Reagents and antibodies

Reagents used were obtained from Sigma (Poole, UK) unless otherwise indicated. BALB/c mice (8-weeks old) were from Harlan Olac (Bicester, UK). Antibodies against murine IL-12 [anti-IL-12 p40 antibody pair and anti-IL-12 p70 enzyme-linked immunosorbent assay (ELISA) set] were obtained from BD PharMingen (San Diego, CA). Anti-phospho-Erk1/2, anti-Erk1/2, anti-phospho-p38, anti-p38 MAPkinase, anti-phospho-thr308PKB, anti-PKB and horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG) were obtained from New England Biolabs (Herts., UK). PD 98059, SB 203580, LY 294002 and wortmannin were from Alexis Biochemicals (San Diego, CA) and U0126 was from Promega (Madison, WI). Anti-IRF-1 and anti-I-κBβ were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Purified ES-62 from *A. viteae* was prepared as described previously.[18]

Purification of murine peritoneal Mϕs, cell culture and cytokine measurement

Thioglycollate-elicited peritoneal Mϕs were prepared as described previously.[19] Cells were cultured in 96-well plates or in small flasks and rested overnight prior to stimulation with 100 U/ml IFN-γ + 100 ng/ml *Salmonella minnesota* LPS (Sigma). Inhibitor studies were performed by preincubation with MAPKinase (MEK), p38 and phosphoinositide 3-kinase (PI 3-kinase) inhibitors (PD 98059 and U0126, SB 203580, LY 294002 and wortmannin, respectively) for 1 hr prior to stimulation with IFN-γ + LPS. Culture supernatants were collected at the time-points indicated and assayed for cytokines by ELISA. Total protein and RNA were purified from cells as described below.

MTT assay

Following removal of culture supernatants for cytokine analysis, cell viability was assessed by replacing medium and adding 500 μg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma). After incubation for 3 hr at 37°, all medium was removed and the precipitate formed in metabolically active cells by the reduction of MTT to its insoluble MTT-formazan was dissolved in isopropanol, and the absorbance (*A*) at 570 nm was determined.

Total protein purification and Western blotting

Cell lysates were prepared as described previously[12] and protein concentrations were determined using the MicroBCA Protein Assay Reagent (Pierce, Rockford, IL). Cell lysates were resolved using the NuPAGE Bis-Tris system (Invitrogen, Paisley, UK) and transferred to nitrocellulose according to the manufacturer's instructions. Nitrocellulose filters were then incubated with wash buffer [25 mM Tris, pH 7·5, 500 mM NaCl, 0·1% (vol/vol) Tween-20] containing 5% milk protein for at least 1 hr to block non-specific protein binding. Primary antibodies were diluted 1 : 1000 in wash buffer containing 5% milk protein and applied to the filter for 1 hr at room

temperature or overnight at 4°. Following washing, the blots were incubated with HRP-conjugated anti-rabbit IgG (diluted 1 : 2000 in wash buffer containing 5% milk protein) for 1 hr at room temperature. Immunoreactive bands were visualized by the enhanced chemiluminescence (ECL) system (Amersham, Bucks., UK).

TaqMan real-time PCR

TaqMan real-time reverse transcription–polymerase chain reaction (RT–PCR) was performed as described previously.[19] The probes used were 5′ FAM (6-carboxy-fluorescein; reporter) and 3′ TAMRA (6-carboxy-tetramethyl rhodamine; quencher). Murine IL-12 p40: Probe 5′-FAM-AACAAGACTTTCCTGA-AGTGTGAAGCACCAAAT-TAMRA-3′, Forward Primer 5′-GGAATTTGGTCCACTGAAATTTTAAA-3′, Reverse Primer 5′-CACGTGAACCGTCCGGAGTA-3′. Murine IL-12 p35: Probe 5′-FAM-CAGCACATTGAAGACCTGTTTACCACTG-GA-TAMRA-3′, Forward Primer 5′-AAGACATCACACGG-GACCAAA-3′, Reverse Primer 5′-CAGGCAACTCTCGTTC-TTGTGTA-3′. Murine TLR4: Probe 5′-FAM-GCCAATTTTG-TCTCCACAGCCACCA-TAMRA-3′, Forward Primer 5′-AGG-AAGTTTCTCTGGACTAACAAGTTTAGA-3′, Reverse Primer 5′-AAATTGTGAGCCACATTGAGTTTC-3′. Each PCR amplification was performed in triplicate, data were analysed using the Applied Biosystems Sequence Detection Software (Applied Biosystems, Warrington, UK) and samples were normalized by their reference reporter hypoxanthine-guanine phosphoribosyltransferase (HPRT).

Statistics

Statistical significance of ELISA data was analysed using the Student's *t*-test.

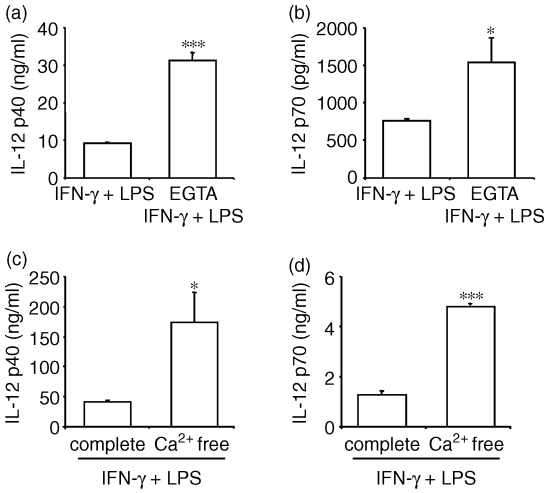

Figure 1. Regulation of interleukin (IL)-12 production by calcium influx. Peritoneal macrophages were pretreated with 5 mM EGTA (a and b) or cultured in calcium-free medium with (complete) or without (Ca^{2+} free) 2 mM $CaCl_2$ to restore normal Ca^{2+} levels (c and d) for 1 hr prior to stimulation with 100 U/ml interferon-γ (IFN-γ) and 100 ng/ml *Salmonella minnesota* lipopolysaccharide (LPS) for 24 hr. IL-12 p40 (monomer, homodimer, p70 heterodimer) and bioactive p70 protein levels in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) (data are presented as mean value plus standard deviation, $n = 3$, $*P < 0{\cdot}05$ $***P < 0{\cdot}005$). Data are representative of four experiments.

RESULTS

Calcium influx negatively regulates the induction of bioactive IL-12

Transient calcium fluxes derived either from intracellular stores or by influx from the extracellular environment have been shown to regulate pro-inflammatory signalling. For example, Sutterwala *et al.*[20] reported that extracellular calcium influx following ligation of Mϕ Fcγ, complement or scavenger receptors results in inhibition of LPS-induced IL-12 production. We therefore investigated the effect of calcium on IFN-γ + LPS-mediated induction of IL-12 p40 and/or p35. Mϕs were cultured in normal medium until 1 hr prior to stimulation with IFN-γ + LPS, when extracellular calcium was depleted either by addition of the calcium chelator, EGTA, or by replacement of the normal medium with calcium-free medium. Stimulation of peritoneal Mϕs with IFN-γ + LPS in the presence of EGTA resulted in an enhanced induction of both p40 and bioactive p70 (Fig. 1a, 1b). Similarly, Mϕs stimulated in calcium-free medium produced more IL-12 (p40 and p70) than those cultured in calcium-free medium where the calcium was restored to normal levels by the addition of calcium chloride (Fig. 1c, 1d). Together, these results suggest that calcium influx negatively regulates IL-12 production and raise the possibility that both subunits may be targeted.

The induction of mRNA encoding the p40 and p35 subunits of bioactive IL-12 following stimulation of peritoneal Mϕs with IFN-γ + LPS was assessed by real-time PCR (TaqMan). An increase in the levels of p40 and p35 mRNA was detectable by 4 hr, maximal by about 8 hr and subsequently returned towards basal levels (Fig. 2a, 2b). As the same threshold was used to obtain Ct values (threshold cycle, i.e. PCR cycle number at which an increase in fluorescence above background levels is first detected) for both p40 and p35, and assuming similar amplification efficiencies/profiles of both probe and primer sets, it is possible to compare the mRNA levels of the two subunits.[21] p40 mRNA appears to be approximately 10-fold more abundant than p35 mRNA, a finding consistent with the fact that these cells produce around 10-fold more p40 protein than p70 (see Fig. 1). Moreover, this finding suggests that the formation of bioactive IL-12 is limited by the upregulation of p35 production. p40 and, to a lesser extent, p35 mRNA levels were both found to be enhanced in Mϕs stimulated in calcium-free medium (Fig. 2c, 2d), indicating that calcium influx was regulating bioactive IL-12 generation at the level of transcription/message stability. To rule out the possibility that calcium influx was simply targeting availability of the LPS receptor, TLR4 mRNA levels were also measured by real-time PCR. Neither culture nor stimulation in the presence of calcium reduced the TLR4 levels of mRNA (Fig. 2e). Moreover, an MTT assay indicated that the viability of Mϕs was not altered by culture in the presence/absence of calcium for the duration of the experiment (Fig. 2f).

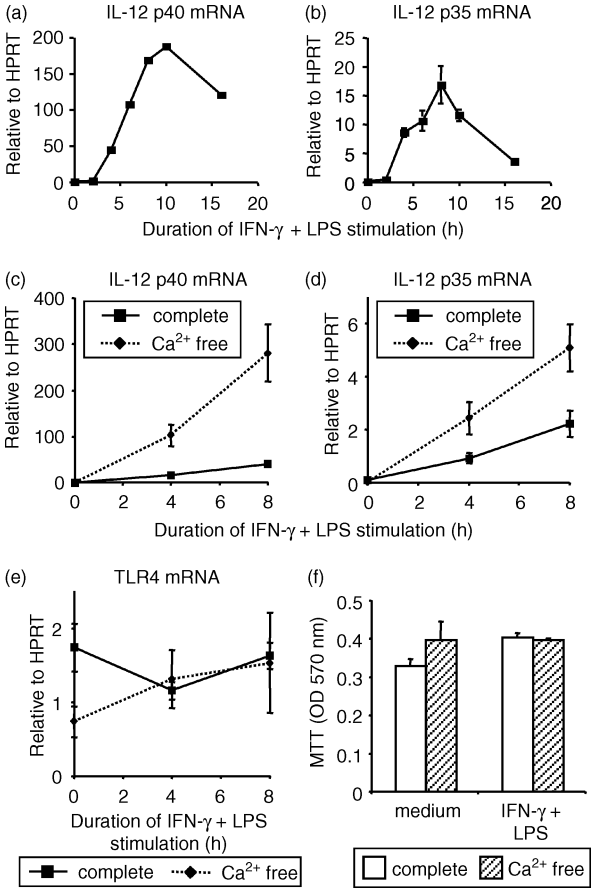

Figure 2. Regulation of interleukin (IL)-12 p40 and p35 mRNA induction by calcium influx. Peritoneal macrophages were cultured in calcium-free medium with (complete) or without (Ca^{2+} free) 2 mM $CaCl_2$ to restore normal Ca^{2+} levels for 1 hr prior to stimulation with 100 U/ml interferon-γ (IFN-γ) and 100 ng/ml *Salmonella minnesota* lipopolysaccharide (LPS) for the times indicated (a–e) or 24 hr (f). IL-12 p40 and p35 and Toll-like receptor 4 (TLR4) mRNA levels were assessed by TaqMan real-time reverse transcription–polymerase chain reaction (RT–PCR), using hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA as a reference (a–e). Cell viability following treatment was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (f). Data are representative of three experiments.

PI 3-kinase negatively regulates the induction of both subunits of IL-12

The conversion of $PI_{4,5}P_2$ to $PI_{3,4,5}P_3$, catalysed by PI 3-kinase, and the resultant recruitment of Tec kinases, such as Btk, appears to be required for sustained activation of phospholipase C and increased calcium fluxes in immune cells.[22–27] Stimulation of peritoneal Mϕs with IFN-γ + LPS resulted in the activation of PI 3-kinase, as indicated by phosphorylation of the downstream target of PI 3-kinase, Akt (also known as protein kinase B, PKB), on Thr308 (Fig. 3a). Pretreatment with the PI 3-kinase inhibitor, LY 294002, enhanced production of the p40 subunit, as well as bioactive IL-12 p70, in a dose-dependent manner (Fig. 3b–3e). Moreover, pretreatment with LY 294002 enhanced the induction of both p40 and p35 mRNA (Fig. 3f, 3g), indicating that PI 3-kinase negatively regulates IL-12 production by targeting the expression of both subunits at the level of transcription/message stability. Similar results were obtained using another PI 3-kinase inhibitor, wortmannin, and neither inhibitor altered the viability of treated cells, as determined by the MTT assay (Fig. 3h and results not shown). Although these results are consistent with PI 3-kinase acting upstream of calcium mobilization, it was also possible that PI 3-kinase was acting in a calcium-independent manner.

Calcium influx, but not PI 3-kinase activity, regulates Erk MAPkinase activation

We have previously shown that Erk MAPkinase negatively regulates LPS-mediated induction of IL-12.[12] Calcium and/or PI 3-kinase signalling has been implicated in the activation of the Erk pathway in many systems.[28–33] Therefore, it was considered possible that PI 3-kinase and/or calcium influx signalling was transducing the negative regulation of IL-12 via Erk MAPkinases. To address this, we investigated whether depletion of extracellular calcium or pretreatment with PI 3-kinase inhibitors reduced the IFN-γ + LPS-stimulated activation of Erk MAPkinase. Indeed, Mϕs stimulated with IFN-γ + LPS in the presence of EGTA (Fig. 4a) or calcium-free medium (results not shown) had reduced levels of dual-phosphorylated Erk1/2 compared with cells stimulated in the presence of calcium. In contrast, LY 294002 pretreatment did not alter IFN-γ + LPS-induced Erk1/2 dual phosphorylation (Fig. 4b), indicating that negative regulation of IL-12 production by PI 3-kinase is Erk-independent and that LPS-induced PI 3-kinase activation can mediate calcium- and Erk-independent signals.

Regulation of bioactive IL-12 p70 induction by p38 and Erk MAPkinases

The finding that PI 3-kinase signalling could negatively regulate IL-12 in an Erk-independent manner led us to reassess the MAPkinase regulation of bioactive IL-12 production. Using the p38 inhibitor, SB 203580, we have previously shown that signals mediated by p38 are required for the induction of IL-12 p40 mRNA and protein following stimulation of Mϕs with LPS.[12] In contrast, PD 98059 treatment to inhibit MEK, the upstream regulator of Erk, showed that these MAPkinases, as alluded to earlier, negatively regulate p40 induction. We therefore performed similar experiments to determine whether p38 and Erk MAPkinases also regulate the induction of the p35 subunit and the formation of bioactive IL-12 p70, using optimal concentrations of inhibitors established previously.[12]

As shown previously by Northern blot analysis,[12] whilst pretreatment of peritoneal Mϕs with 5 μM SB 203580 for 1 hr prior to IFN-γ + LPS stimulation reduced the production of p40 mRNA levels, as assessed by real-time RT–PCR, 20 μM PD 98059 enhanced IL-12 p40 mRNA levels at late time-points (Fig. 5a). This is a finding consistent with Erk MAPkinase acting as a negative feedback regulator. These effects are also reflected at the IL-12 p40 protein level (Fig. 5b). We then showed that pretreatment with 5 μM SB 203580 also reduces

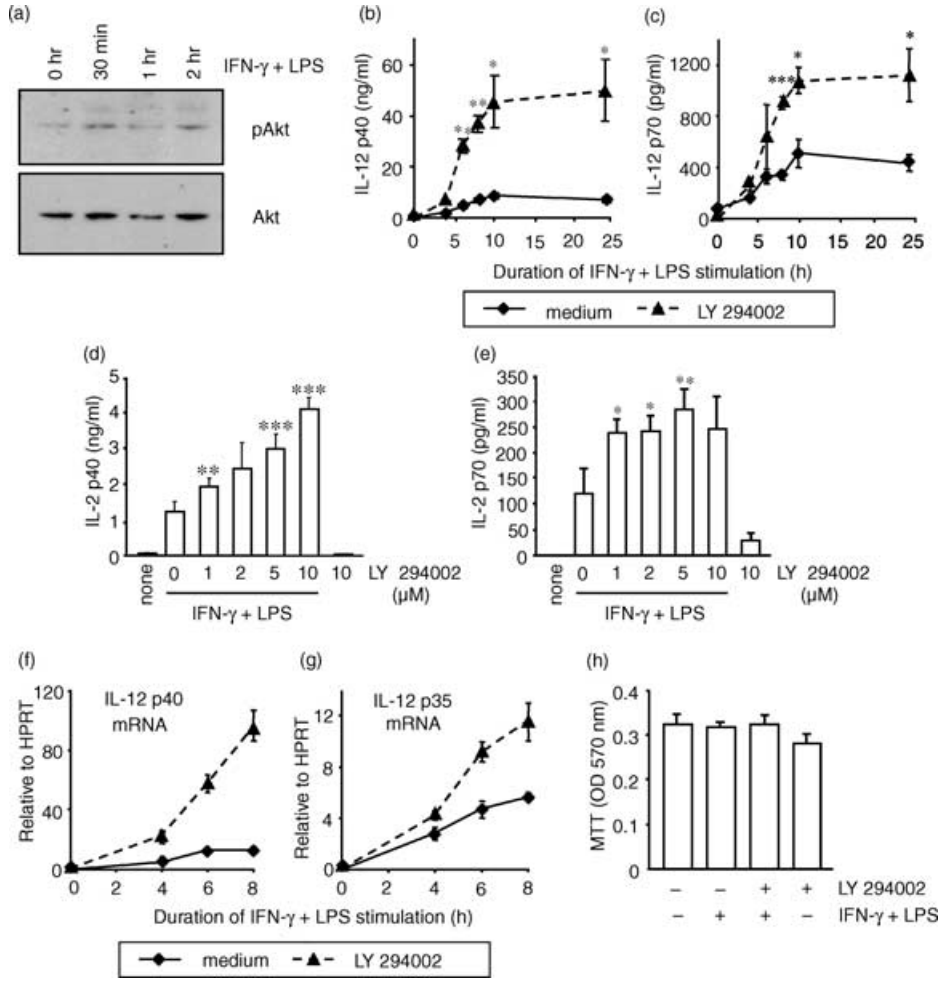

Figure 3. Regulation of interleukin (IL)-12 induction by phosphoinositide 3-kinase (PI 3-kinase). (a) Peritoneal macrophages (Mϕs) were stimulated with 100 U/ml interferon-γ (IFN-γ) and 100 ng/ml *Salmonella minnesota* lipopolysaccharide (LPS) for the times indicated. Thr308-phosphorylated Akt (pAkt) and total Akt (Akt) protein levels in cell lysates were assessed by Western blotting. (b–h) Peritoneal Mϕs were pretreated with the PI 3-kinase inhibitor LY 294002 (b, c and f–h 10 μM; d and e at the concentrations indicated) for 1 hr prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml *S. minnesota* LPS for the times indicated (b, c, f and g) or 24 hr (d, e and h). Levels of IL-12 p40 (monomer, homodimer, p70 heterodimer) and bioactive p70 protein in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) (b, c, d and e; data presented as mean value plus standard deviation, $n = 3$, $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}005$ compared with IFN-γ + LPS stimulation in the absence of LY 294002). IL-12 p40 and p35 mRNA levels were assessed by TaqMan real-time reverse transcription–polymerase chain reaction (RT–PCR), using hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA as a reference (f and g). Cell viability following treatment was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (h). Data are representative of three experiments.

the production of p35 mRNA levels (Fig. 5c), indicating that p38 MAPkinase signals are required for the induction of both subunits and hence bioactive IL-12 generation. In contrast, pretreatment with 20 μM PD 98059 did not significantly alter p35 mRNA levels (Fig. 5c), indicating that p35 induction is Erk-independent. Furthermore, while p40 induction (mRNA and protein) was enhanced, release of bioactive p70 was not altered by pretreatment with PD 98059 (Fig. 5d). In contrast, pretreatment with SB 203580 reduced the induction of both p40 and p70 protein (Fig. 5e), consistent with the requirement of p38 MAPkinase for the induction of both the p40 and p35 subunits (Fig. 5a, 5c). Taken together, these results indicate that the levels of bioactive IL-12 p70 production are predominantly controlled by p35 availability rather than being subject to Erk-mediated negative feedback regulation.

Calcium influx signals regulate IL-12 production by targeting IRF-1 and NF-κB

The IRF family members IRF-1, IRF-2 and interferon consensus binding protein (ICSBP) have been identified as components of the transcription factor complexes that regulate IL-12 p40 and p35 promoter activity.[34,35] Indeed, IL-12 p40 production is reduced in Mϕs from IRF-1 and IRF-2 knockout mice.[36–38] IRF-1 may also be required for p35 induction, although this is controversial.[37,38] Stimulation of peritoneal Mϕs with

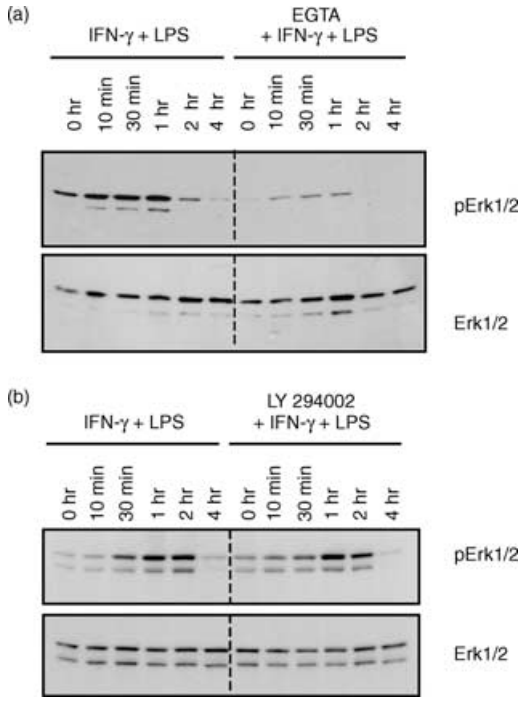

Figure 4. Regulation of Erk mitogen-activated protein kinase (MAP-kinase) induction by calcium influx and phosphoinositide 3-kinase (PI 3-kinase). Peritoneal macrophages were pretreated with EGTA (5 mM; a) or the PI 3-kinase inhibitor, LY 294002 (10 μM; b), for 1 hr prior to stimulation with 100 U/ml interferon-γ (IFN-γ) and 100 ng/ml *Salmonella minnesota* lipopolysaccharide (LPS) for the times indicated. Dual-phosphorylated Erk1/2 (pErk1/2) and total Erk1/2 (Erk1/2) protein levels in cell lysates were assessed by Western blotting. Data are representative of three experiments.

IFN-γ + LPS induced the synthesis of IRF-1, whereas IRF-2 was detected in resting cells and did not change following stimulation (Fig. 6a). Thus, reduced IRF-1 induction could provide a mechanism for the reduction in IL-12 induction by calcium influx. Indeed, the kinetics of IRF-1 synthesis were altered in the absence of calcium (Fig. 6b). IRF-1 levels were enhanced at 1- and 2-hr time-points, suggesting that calcium influx suppresses IRF-1 production and hence IL-12 p40 and/or p35 production. Levels of IRF-2 were not modulated by the presence or absence of calcium (results not shown).

NF-κB activation has also been shown to be important for the regulation of IL-12 production.[39–42] We therefore assessed the effects of calcium influx on NF-κB activation, as evidenced by degradation of its negative regulator I-κB. Indeed, I-κBβ was found to be degraded more rapidly in response to stimulation with IFN-γ + LPS in the absence of calcium (Fig. 6b). These results indicate that calcium influx may also regulate IL-12 production by targeting NF-κB signalling.

In contrast, pretreatment with LY 294002 did not significantly alter either IRF-1/IRF-2 synthesis or I-κBβ degradation (results not shown), indicating that PI 3-kinase does not regulate IL-12 p40 production by targeting either of these transcription factors. Moreover, taken together with the Erk MAPkinase data (Fig. 4b) these results suggest that PI 3-kinase signals to suppress IL-12 in a manner independent of calcium influx in Mϕs stimulated with IFN-γ + LPS.

Erk MAPkinase targets IRF-1, but not NF-κB, to achieve suppression of IL-12 p40 gene expression

To determine whether calcium influx modulated IRF-1 and NF-κB in an Erk MAPkinase-dependent manner, we determined whether pretreatment with PD 98059 elevated IRF-1 synthesis. We have shown, by Western blotting, that IRF-1 synthesis was maximal by 2–4 hr after stimulation (Fig. 6a) and, at 4 hr, the IRF-1 levels were indeed elevated in Mϕs that had been pretreated with PD 98059 (Fig. 6c). These results suggest that Erk MAPkinase can negatively regulate IL-12 p40 production by mediating calcium-driven suppression of IRF-1 synthesis. By contrast, we have previously demonstrated that inhibition of Erk does not alter NF-κB binding to the IL-12 p40 promoter in the murine Mϕ cell line J774.[12] Consistent with this we see similar levels of I-κBβ degradation in control and PD 98059-treated cells (Fig. 6c). Interestingly, SB 203580 does not appear to mediate its inhibitory effects on IL-12 production either by suppressing IRF-1 expression or preventing I-κBβ degradation (Fig. 6c). These results are again consistent with our previous findings that inhibition of p38 MAPkinase did not block NF-κB or IRF-1 binding to the IL-12 p40 promoter.[12] The finding that calcium influx suppresses both IRF-1 expression and I-κB degradation, whilst Erk MAPkinase only inhibits IRF-1, may explain why Erk MAPkinase only targets IL-12 p40, whilst calcium influx ablates both IL-12 p40 and p35 and hence bioactive IL-12 p70 induction.

Filarial nematode products can inhibit IL-12 p40 production by Erk-dependent mechanisms

We have previously demonstrated that a phosphorylcholine-containing excretory-secretory (ES) product of the filarial nematode *A. viteae*, ES-62, inhibits the induction of IL-12 (p40 and p70) by IFN-γ + LPS.[19] We now show that ES-62-mediated suppression of IL-12 p40 is probably mediated by Erk MAPkinase, as the inhibition of p40 production by ES-62 can be rescued by pretreatment with PD 98059 (Fig. 7a, 7b). In contrast, p70 production is only very slightly restored by pretreatment with PD 98059 (Fig. 7b), indicating that although PD 98059 treatment counteracts the ES-62-mediated suppression of p40 induction, it is not able to restore p35, and therefore p70, heterodimer levels. These findings are consistent with our demonstration that p35 induction is not regulated by Erk MAPkinase (Fig. 5) and our proposal that p35, rather than p40, availability plays the dominant role in determining the levels of bioactive IL-12 p70 production. Moreover, these data indicate that this pathogen product employs other signalling mechanisms to downregulate IL-12 p35. Indeed, we have found that pretreatment with ES-62 acts to reduce IFN-γ + LPS-stimulated activation of p38 MAPkinase (Fig. 7c) and because p38 MAPkinase activation is essential for p35 upregulation (Fig. 5), this may explain the Erk-independent suppression of p35, and hence bioactive IL-12 p70, by ES-62.

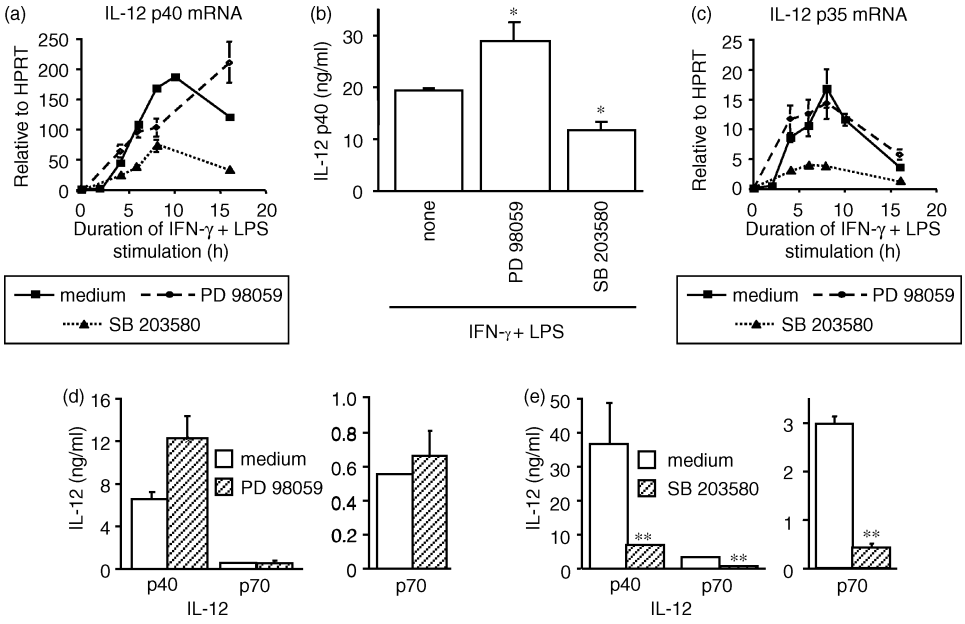

Figure 5. The role of Erk and p38 mitogen-activated protein kinase (MAPkinase) in the induction of interleukin (IL)-12 subunits and bioactive IL-12. Peritoneal macrophages (Mϕs) were pretreated with 20 μM PD 98059 or 5 μM SB 203580 for 1 hr prior to stimulation with 100 U/ml interferon-γ (IFN-γ) and 100 ng/ml *Salmonella minnesota* lipopolysaccharide (LPS) for the times indicated (a and c) or 24 hr (b, d and e). IL-12 p40 and p35 mRNA levels were assessed by TaqMan real-time reverse transcription–polymerase chain reaction (RT–PCR) using hypoxanthine-guanine phosphoribosyltransferase (HPRT) as a reference (a and c). Basal levels of IL-12 p40 and p35 mRNA remain constant in unstimulated peritoneal Mϕs. IL-12 p40 (monomer, homodimer, p70 heterodimer) and bioactive p70 protein levels in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) (b, d and e, with p70 data expanded in d and e; data presented as mean value plus standard deviation, $n = 3$, $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ compared with IFN-γ + LPS stimulation in the absence of PD 98059 or SB 203580). Basal levels of IL-12 production by unstimulated Mϕs were < 200 pg/ml of p40 and < 20 pg/ml of p70 (d and e); neither PD 98059 nor SB 203580 treatment altered the basal levels of p40 or p70. Data are representative of three experiments.

DISCUSSION

Bioactive IL-12 is a 70 000-MW heterodimeric cytokine, comprising p40 and p35 subunits, and we now propose that p35 induction is the limiting factor in the generation of bioactive IL-12. This proposal is consistent with our finding that IL-12 p40 mRNA and protein is found in 10-fold excess of that of p35 mRNA and p70 protein. Moreover, it makes biological sense to regulate bioactive IL-12 p70 at the level of p35 transcription, given that p40 can form homodimers that antagonize bioactive IL-12[4,5] and also heterodimerize with p19 to form the novel cytokine IL-23, which exhibits overlapping, yet distinct, functions to those of IL-12.[2,3] Consistent with this, we also show that differential regulatory mechanisms exist for the induction of the p40 and p35 subunits of the bioactive IL-12 heterodimer (summarized in Fig. 8), which probably provide therapeutic targets for precise and specific fine-tuning of IL-12/IL-23-related responses necessary to combat infection by pathogens or pathology resulting from inflammatory disorders.

Blocking of calcium influx using EGTA had previously been shown to prevent suppression of IL-12 p40 production following ligation of Mϕ Fcγ, complement or scavenger receptors, without affecting other cytokines such as IL-10 and tumour necrosis factor-α (TNF-α).[20] These data suggest that calcium influx selectively negatively regulates IL-12 production. We confirmed that calcium influx inhibits IL-12 p40 production and have extended this to show that the generation of bioactive IL-12 p70 is similarly suppressed by targeting p40 and p35 induction at the mRNA level, possibly by modulating activation of IRF-1 and NF-κB. As, in contrast to p40, p35 gene expression is cyclohexamide-independent and hence only requires pre-synthesized activators,[11] it is probable that the calcium-mediated suppression of IRF-1 expression selectively targets p40 induction (Fig. 8). Nevertheless, activation of pre-existing IRF-1 may still be necessary for IFN-γ + LPS stimulation of p35. However, although it was previously reported that IRF-1 is required,[37] a recent report suggested that such p35 induction is IRF-1-independent.[38]

Given that we had previously shown that Erk MAPkinase also negatively regulated LPS-induced IL-12 p40 generation,[12] and that calcium signalling has been implicated in the regulation of Erk MAPkinase activation in many systems,[28–30] we investigated whether the effects of calcium influx were mediated by Erk MAPkinases. We indeed found that calcium influx stimulated Erk activity, and that Erk MAPkinases negatively regulate IRF-1 induction, suggesting that calcium-mediated suppression of IL-12 p40 may be Erk- and IRF-1-dependent (Fig. 8). However, we previously reported that Erk MAPkinase does not negatively regulate LPS induction of TNF-α.[12] As we now show that Erk MAPkinases mediate (at least some of) the

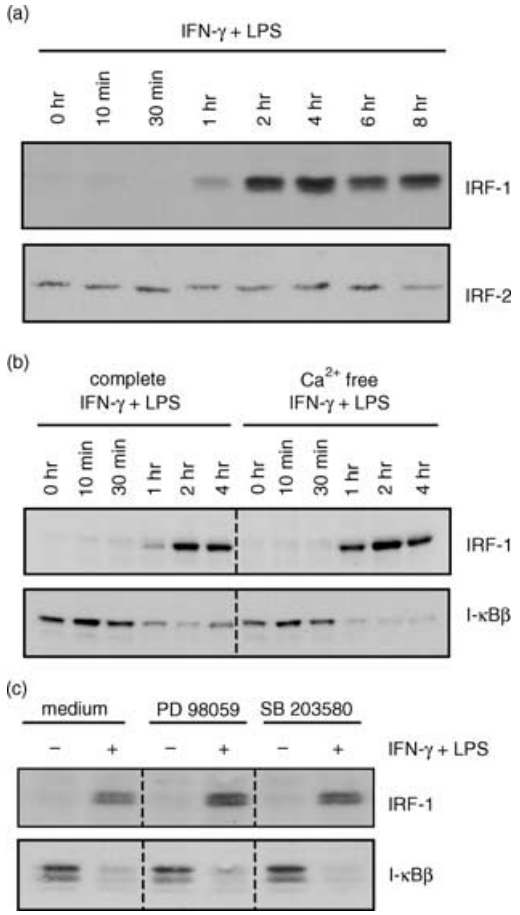

Figure 6. Regulation of interferon regulatory factor-1 (IRF-1) and nuclear factor (NF)-κB signalling pathways by calcium influx and Erk mitogen-activated protein kinases (MAPkinases). (a) and (b) Peritoneal macrophages (Mϕs) were cultured in normal medium (a), or in calcium-free medium with (complete) or without (Ca^{2+} free) addition of 2 mM $CaCl_2$ to restore normal Ca^{2+} levels (b), for 1 hr prior to stimulation with 100 U/ml interferon-γ (IFN-γ) and 100 ng/ml *Salmonella minnesota* lipopolysaccharide (LPS) for the times indicated. (c). Peritoneal Mϕs were pretreated with 20 μM PD 98059 or 5 μM SB 203580 for 1 hr prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml *S. minnesota* for 4 hr. IRF-1, IRF-2 and I-κBβ protein levels in cell lysates were assessed by Western blotting. Data are representative of three experiments.

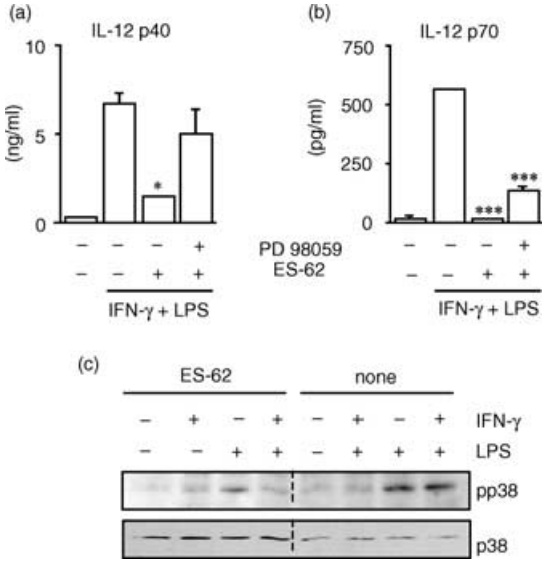

Figure 7. Erk-mediated regulation of interleukin (IL)-12 production by a filarial nematode product. (a) and (b) Peritoneal macrophages (Mϕs) were pretreated with 20 μM PD 98059 for 1 hr prior to treatment with 2 μg/ml excretory-secretory (ES)-62 for 18 hr, followed by stimulation with 100 U/ml interferon-γ (IFN-γ) and 100 ng/ml *Salmonella minnesota* lipopolysaccharide (LPS) for 24 hr. IL-12 p40 (monomer, homodimer, p70 heterodimer) and bioactive p70 protein levels in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) (data are presented as mean value plus standard deviation, $*P < 0{\cdot}05$, $***P < 0{\cdot}005$ compared with IFN-γ + LPS stimulation in the absence of PD 98059 and ES-62). (c). Peritoneal Mϕs were pretreated with 2 μg/ml ES-62 for 18 hr prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml *S. minnesota* LPS for 10 min. Dual-phosphorylated p38 (pp38) and total p38 (p38) mitogen-activated protein kinase (MAPkinase) levels in cell lysates were assessed by Western blotting. Data are representative of three experiments.

downstream effects of calcium influx, this is consistent with the report by Sutterwala *et al*.[20] that whilst calcium influx inhibited LPS induction of IL-12 p40, it did not block LPS induction of TNF-α. Similarly, we have also shown that a synthetic version of the repeating disaccharide component of *Leishmania* lipophosphoglycan (LPG) suppresses the induction of IL-12 p40 by IFN-γ + LPS, at least in part, by enhancing the activation of Erk MAPkinases.[12] *Leishmania* infection of monocytes has been shown to result in elevated intracellular calcium levels owing to the increased permeability of plasma membrane Ca^{2+} channels[43] and this could therefore provide a link to the Erk-mediated suppression of IL-12 production by LPG.

Although calcium influx can negatively regulate the induction of both p35 and p40, by contrast, we discovered that Erk signalling did not modulate p35 induction, suggesting that calcium influx modulates bioactive IL-12 production by Erk-dependent and -independent mechanisms (Fig. 8). Moreover, the finding that calcium influx inhibits I-κB degradation, whilst Erk MAPkinase signalling does not suppress NF-κB activation,[12] suggests that the effects of calcium influx on NF-κB are Erk-independent and may be targeted towards p35 induction.

PI 3-kinase has also been implicated in the negative regulation of expression of inflammatory genes such as inducible nitric oxide synthase (iNOS) and TNF-α.[44,45] Interestingly, LPS stimulation has been shown to result in the activation of PI 3-kinase.[44,46] Such PI 3-kinase activation generates the lipid second messenger, $PI_{3,4,5}P_3$, leading to the membrane association and sustained activation of phospholipase C, resulting in calcium influx and activation of lipid-dependent kinases such as protein kinase B/Akt (reviewed in refs 47 and 48). We therefore investigated whether PI 3-kinase mediated these negative regulatory effects via calcium influx and/or Erk MAPkinase activation. Although, like calcium influx, PI 3-kinase negatively regulated the induction of p40 and p35, it appeared to do this in an Erk-, IRF-1- and NF-κB-independent manner. Moreover, the finding that PI 3-kinase negatively regulates TNF-α production[44,45] supports our proposal that the effects of PI 3-kinase

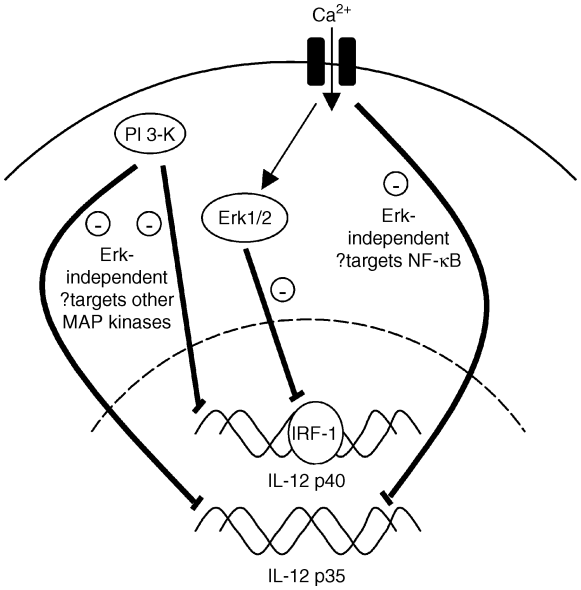

Figure 8. Summary of calcium influx- and phosphoinositide 3-kinase (PI 3-kinase)-dependent mechanisms of negative regulation of interleukin (IL)-12 p40 and p35 gene induction. The induction of both subunits of bioactive IL-12 p70 is negatively regulated by independent calcium influx and PI 3-kinase (PI 3-K) pathways. Calcium influx suppresses interferon regulatory factor-1 (IRF-1) induction via an Erk mitogen-activated protein kinase (MAPkinase)-dependent mechanism. Erk-independent negative regulation of p35 may occur via suppression of the nuclear factor (NF)-κB pathway. PI 3-kinase-mediated suppression of both p40 and p35 is Erk-independent, but may act via suppression of other MAPkinases [p38, c-Jun N-terminal protein kinase (JNK)].

occur in a manner independent of calcium influx and Erk MAPkinase activation (Fig. 8). Although a recent study using human monocytes suggested that such PI 3-kinase regulation is mediated via MAPkinases,[45] our data suggest that PI 3-kinase may achieve its inhibitory effects on IL-12 by targeting the other MAPkinase subfamilies [p38 and c-Jun N-terminal protein kinase (JNK)] that are required for IL-12 induction.[12,49,50] Indeed, Fukao *et al*. recently demonstrated such a negative regulatory role for PI 3-kinase in the LPS induction of p40, p35 and bioactive p70 IL-12 by showing that LPS-stimulated p38 activity was enhanced by wortmannin treatment and in bone marrow-derived DCs from PI 3-kinase knockout mice.[51]

In contrast to the negative regulation of IL-12 exhibited by calcium, PI 3-kinase and Erk MAPkinase signalling described here, we show that p38 MAPkinase activation is essential for the induction of both p40 and p35 and hence bioactive IL-12. p35 shares some homology with IL-6 and granulocyte–colony-stimulating factor (G-CSF),[52] while p40 is homologous to the IL-6 and G-CSF receptors.[53,54] Interestingly, we have found that IL-6 induction is also p38-dependent, but Erk-independent (data not shown), indicating that similar regulatory pathways signal the induction of these related cytokines and supporting the notion that bioactive IL-12 may have been derived from a primordial cytokine and its receptor.

The filarial nematode product, ES-62, can polarize immune responses to an anti-inflammatory phenotype associated with parasite survival (reviewed in ref. 55). Consistent with this, we have previously shown that pre-exposure to ES-62 renders Mϕs refractory to subsequent induction of bioactive IL-12 p70 by IFN-γ + LPS by targeting the induction of mRNA encoding both subunits.[19] We now show that this suppression of IL-12 p40, but not p35, can be prevented by the MEK inhibitor, PD 98059, suggesting that ES-62 can activate the Erk-dependent negative feedback pathway to suppress production of IL-12. However, activation of this Erk pathway alone does not fully explain the effects of ES-62, as bioactive IL-12 production is limited by p35 induction. Consistent with this, exposure to ES-62 was also found to suppress the IFN-γ + LPS-mediated activation of p38 MAPkinase that is essential for induction of both p40 and p35. Taken together, these results suggest that ES-62 suppresses bioactive IL-12, primarily by inhibiting p38 MAPkinase activity. However, ES-62 may also employ the Erk-dependent negative regulatory pathway to reduce the availability of IL-12 p40, not only for limiting generation of bioactive IL-12 but also for the related, yet distinct cytokine, IL-23. In any case, that filarial nematodes produce a molecule that subverts both signalling pathways can only emphasize that each has an important role in regulating IL-12 responses.

In conclusion, it has recently emerged that IL-12 p40 and p35 subunits are components of cytokines/immunomodulators other than bioactive IL-12 p70,[2,3] for example, IL-12 p40 homodimers, IL-23 p40p19 and EBI-3p35.[56] Moreover, emerging data demonstrate that such redundancy in the IL-12/IL-23/IL-27 family extends to the role of these subunits in eliciting autoimmune disease pathogenesis, with IL-12 p40 monomers being associated with systemic lupus erythematosus (SLE) disease activity,[57] IL-23 p40p19 heterodimers being implicated in experimental autoimmune encephalomyelitis (EAE),[58] and the overexpression of IL-23 p19 resulting in multiorgan inflammation and premature death.[59] Therefore, the relative kinetics and levels of induction of these individual cytokine subunits must play a key role in determining the precise phenotype of the resulting immune response and/or disease pathogenesis. Delineation of the differential regulatory pathways involved will therefore identify novel targets for immunomodulation at the level of individual IL-12-related cytokines and their specific functional responses.

ACKNOWLEDGMENTS

This work was supported by the Wellcome Trust and the Biotechnology and Biological Sciences Research Council.

REFERENCES

1 Ma X, Trinchieri G. Regulation of interleukin-12 production in antigen-presenting cells. Adv Immunol 2001; **79:**55–92.
2 Oppmann B, Lesley R, Blom B *et al.* Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 2000; **13:**715–25.
3 Belladonna ML, Renauld JC, Bianchi R *et al.* IL-23 and IL-12 have overlapping, but distinct, effects on murine dendritic cells. J Immunol 2002; **168:**5448–54.

4 Gillessen S, Carvajal D, Ling P *et al.* Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. Eur J Immunol 1995; **25:**200–6.
5 Ling P, Gately MK, Gubler U *et al.* Human IL-12 p40 homodimer binds to the IL-12 receptor but does not mediate biologic activity. J Immunol 1995; **154:**116–27.
6 Devergne O, Birkenbach M, Kieff E. Epstein–Barr virus-induced gene 3 and the p35 subunit of interleukin 12 form a novel heterodimeric hematopoietin. Proc Natl Acad Sci USA 1997; **94:** 12041–6.
7 Pflanz S, Timans JC, Cheung J *et al.* IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. Immunity 2002; **16:**779–90.
8 Tone Y, Thompson SA, Babik JM, Nolan KF, Tone M, Raven C, Waldmann H. Structure and chromosomal location of the mouse interleukin-12 p35 and p40 subunit genes. Eur J Immunol 1996; **26:**1222–7.
9 Babik JM, Adams E, Tone Y, Fairchild PJ, Tone M, Waldmann H. Expression of murine IL-12 is regulated by translational control of the p35 subunit. J Immunol 1999; **162:**4069–78.
10 Murphy FJ, Hayes MP, Burd PR. Disparate intracellular processing of human IL-12 preprotein subunits: atypical processing of the P35 signal peptide. J Immunol 2000; **164:**839–47.
11 Aste-Amezaga M, Ma X, Sartori A, Trinchieri G. Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10. J Immunol 1998; **160:**5936–44.
12 Feng GJ, Goodridge HS, Harnett MM, Wei XQ, Nikolaev AV, Higson AP, Liew FY. Extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases differentially regulate the lipopolysaccharide-mediated induction of inducible nitric oxide synthase and IL-12 in macrophages: Leishmania phosphoglycans subvert macrophage IL-12 production by targeting ERK MAP kinase. J Immunol 1999; **163:** 6403–12.
13 Weinstein SL, Sanghera JS, Lemke K, Defranco AL, Pelech SL. Bacterial lipopolysaccharide induces tyrosine phosphorylation and activation of mitogen-activated protein-kinases in macrophages. J Biol Chem 1992; **267:**14955–62.
14 Han J, Lee JD, Bibbs L, Ulevitch RJ. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 1994; **265:**808–11.
15 Hambleton J, McMahon M, Defranco AL. Activation of Raf-1 and mitogen-activated protein-kinase in murine macrophages partially mimics lipopolysaccharide-induced signalling events. J Exp Med 1995; **182:**147–54.
16 Hsuan SL, Kannan MS, Jeyaseelan S, Prakash YS, Sieck GC, Maheswaran SK. *Pasteurella haemolytica* A1-derived leukotoxin and endotoxin induce intracellular calcium elevation in bovine alveolar macrophages by different signaling pathways. Infect Immun 1998; **66:**2836–44.
17 Seabra V, Stachlewitz RF, Thurman RG. Taurine blunts LPS-induced increases in intracellular calcium and TNF-alpha production by Kupffer cells. J Leukoc Biol 1998; **64:**615–21.
18 Harnett W, Harnett MM. Inhibition of murine B cell proliferation and down-regulation of protein kinase C levels by a phosphorylcholine-containing filarial excretory-secretory product. J Immunol 1993; **151:**4829–37.
19 Goodridge HS, Wilson EH, Harnett W, Campbell CC, Harnett MM, Liew FY. Modulation of macrophage cytokine production by ES-62, a secreted product of the filarial nematode *Acanthocheilonema viteae*. J Immunol 2001; **167:**940–5.
20 Sutterwala FS, Noel GJ, Clynes R, Mosser DM. Selective suppression of interleukin-12 induction after macrophage receptor ligation. J Exp Med 1997; **185:**1977–85.
21 Overbergh L, Valckx D, Waer M, Mathieu C. Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. Cytokine 1999; **11:**305–12.
22 Tuveson DA, Carter RH, Soltoff SP, Fearon DT. CD19 of B cells as a surrogate kinase insert to bind phosphatidylinositol-3-kinase. Science 1993; **260:**986–9.
23 Yano H, Nakanishi S, Kimura K, Hanai N, Saitoh Y, Fukui Y, Nonomura Y, Matsuda Y. Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. J Biol Chem 1993; **268:**25846–56.
24 Buhl AM, Cambier JC. Co-receptor and accessory regulation of B-cell antigen receptor signal transduction. Immunol Rev 1997; **160:**127–38.
25 Bolland S, Pearse RN, Kurosaki T, Ravetch JV. SHIP modulates immune receptor responses by regulating membrane association of Btk. Immunity 1998; **8:**509–16.
26 Cantrell DA. Phosphoinositide 3-kinase signalling pathways. J Cell Sci 2001; **114:**1439–45.
27 Smith CI, Islam TC, Mattsson PT, Mohamed AJ, Nore BF, Vihinen M. The Tec family of cytoplasmic tyrosine kinases: mammalian Btk, Bmx, Itk, Tec, Txk and homologs in other species. Bioessays 2001; **23:**436–46.
28 Chao TS, Byron KL, Lee KM, Villereal M, Rosner MR. Activation of MAP kinases by calcium-dependent and calcium-independent pathways. Stimulation by thapsigargin and epidermal growth factor. J Biol Chem 1992; **267:**19876–83.
29 Rosen LB, Ginty DD, Weber MJ, Greenberg ME. Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. Neuron 1994; **12:**1207–21.
30 Zohn IE, Yu H, Li X, Cox AD, Earp HS. Angiotensin II stimulates calcium-dependent activation of c-Jun N-terminal kinase. Mol Cell Biol 1995; **15:**6160–8.
31 Grammer TC, Blenis J. Evidence for MEK-independent pathways regulating the prolonged activation of the ERK-MAP kinases. Oncogene 1997; **14:**1635–42.
32 Chang LC, Wang JP. Examination of the signal transduction pathways leading to activation of extracellular signal-regulated kinase by formyl-methionyl-leucyl-phenylalanine in rat neutrophils. FEBS Lett 1999; **454:**165–8.
33 Li X, Carter RH. CD19 signal transduction in normal human B cells: linkage to downstream pathways requires phosphatidylinositol 3-kinase, protein kinase C and Ca^{2+}. Eur J Immunol 2000; **30:** 1576–86.
34 Ma X, Aste-Amezaga M, Gri G, Gerosa F, Trinchieri G. Immunomodulatory functions and molecular regulation of IL-12. Chem Immunol 1997; **68:**1–22.
35 Wang IM, Contursi C, Masumi A, Ma X, Trinchieri G, Ozato K. An IFN-gamma-inducible transcription factor, IFN consensus sequence binding protein (ICSBP), stimulates IL-12 p40 expression in macrophages. J Immunol 2000; **165:**271–9.
36 Lohoff M, Ferrick D, Mittrucker HW, Duncan GS, Bischof S, Rollinghoff M, Mak TW. Interferon regulatory factor-1 is required for a T helper 1 immune response *in vivo*. Immunity 1997; **6:**681–9.
37 Salkowski CA, Kopydlowski K, Blanco J, Cody MJ, McNally R, Vogel SN. IL-12 is dysregulated in macrophages from IRF-1 and IRF-2 knockout mice. J Immunol 1999; **163:**1529–36.
38 Maruyama S, Sumita K, Shen H *et al.* Identification of IFN regulatory factor-1 binding site in IL-12 p40 gene promoter. J Immunol 2003; **170:**997–1001.
39 Murphy TL, Cleveland MG, Kulesza P, Magram J, Murphy KM. Regulation of interleukin-12 p40 expression through an NF-kappa-B half-site. Mol Cell Biol 1995; **15:**5258–67.
40 Gri G, Savio D, Trinchieri G, Ma XJ. Synergistic regulation of the human interleukin-12 p40 promoter by NF kappa B and Ets tran-

scription factors in Epstein–Barr virus-transformed B cells and macrophages. J Biol Chem 1998; **273:**6431–8.
41 Plevy SE, Gemberling JHM, Hsu S, Dorner AJ, Smale ST. Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters. Evidence of functional synergy between C/EBP and Rel proteins. Mol Cell Biol 1997; **17:**4572–88.
42 Sanjabi S, Hoffmann A, Liou HC, Baltimore D, Smale ST. Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages. Proc Natl Acad Sci USA 2000; **97:**12705–10.
43 Olivier M, Baimbridge KG, Reiner NE. Stimulus-response coupling in monocytes infected with Leishmania. Attenuation of calcium transients is related to defective agonist-induced accumulation of inositol phosphates. J Immunol 1992; **148:**1188–96.
44 Park YC, Lee CH, Kang HS, Chung HT, Kim HD. Wortmannin, a specific inhibitor of phosphatidylinositol-3-kinase, enhances LPS-induced NO production from murine peritoneal macrophages. Biochem Biophys Res Commun 1997; **240:**692–6.
45 Guha M, Mackman N. The PI3K-Akt pathway limits LPS activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. J Biol Chem 2002; **6:**6.
46 Lee J, Mira-Arbibe L, Ulevitch RJ. TAK1 regulates multiple protein kinase cascades activated by bacterial lipopolysaccharide. J Leukoc Biol 2000; **68:**909–15.
47 Carpenter CL, Cantley LC. Phosphoinositide kinases. Curr Opin Cell Biol 1996; **8:**153–8.
48 Scheid MP, Woodgett JR. Protein kinases. six degrees of separation? Curr Biol 2000; **10:**R191–4.
49 Hacker H, Mischak H, Miethke T *et al.* CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. EMBO J 1998; **17:**6230–40.
50 Constant SL, Dong C, Yang DD, Wysk M, Davis RJ, Flavell RA. JNK1 is required for T cell-mediated immunity against *Leishmania major* infection. J Immunol 2000; **165:**2671–6.
51 Fukao T, Tanabe M, Terauchi Y *et al.* PI3K-mediated negative feedback regulation of IL-12 production in DCs. Nat Immunol 2002; **3:**875–81.
52 Merberg DM, Wolf SF, Clark SC. Sequence similarity between NKSF and the IL-6/G-CSF family [letter]. Immunol Today 1992; **13:**77–8.
53 Gearing DP, Cosman D. Homology of the p40 subunit of natural killer cell stimulatory factor (NKSF) with the extracellular domain of the interleukin-6 receptor [letter]. Cell 1991; **66:**9–10.
54 Schoenhaut DS, Chua AO, Wolitzky AG *et al.* Cloning and expression of murine IL-12. J Immunol 1992; **148:**3433–40.
55 Harnett W, Harnett M. Immunomodulation by filarial nematode phosphorylcholine-containing glycoproteins. In: Kennedy MW, Harnett W, eds. Parasitic Nematodes: Molecular Biology, Biochemistry and Immunology. Wallingford, UK: CABI Publishing 2001:399–414.
56 Devergne O, Hummel M, Koeppen H, Le Beau MM, Nathanson EC, Kieff E, Birkenbach M. A novel interleukin-12 p40-related protein induced by latent Epstein–Barr virus infection in B lymphocytes. J Virol 1996; **70:**1143–53.
57 Lauwerys BR, Van Snick J, Houssiau FA. Serum IL-12 in systemic lupus erythematosus: absence of p70 heterodimers but presence of p40 monomers correlating with disease activity. Lupus 2002; **11:**384–7.
58 Gran B, Zhang GX, Yu S, Li J, Chen XH, Ventura ES, Kamoun M, Rostami A. IL-12p35-deficient mice are susceptible to experimental autoimmune encephalomyelitis: evidence for redundancy in the IL-12 system in the induction of central nervous system autoimmune demyelination. J Immunol 2002; **169:**7104–10.
59 Wiekowski MT, Leach MW, Evans EW *et al.* Ubiquitous transgenic expression of the IL-23 subunit p19 induces multiorgan inflammation, runting, infertility, and premature death. J Immunol 2001; **166:**7563–70.